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MicroRNA-92a Controls Angiogenesis and Functional Recovery of Ischemic Tissues in Mice

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MicroRNA-92a Controls Angiogenesis and Functional Recovery of Ischemic Tissues in Mice

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MicroRNAs (miRs) are small noncoding RNAs that regulate gene expression by binding to target messenger RNAs (mRNAs), leading to translational repression or degradation. Here, we show that the miR-17~92 cluster is highly expressed in human endothelial cells and that miR-92a, a component of this cluster, controls the growth of new blood vessels (angiogenesis). Forced overexpression of miR-92a in endothelial cells blocked angiogenesis in vitro and in vivo. In mouse models of limb ischemia and myocardial infarction, systemic administration of an antagomir designed to inhibit miR-92a led to enhanced blood vessel growth and functional recovery of damaged tissue. MiR-92a appears to target mRNAs corresponding to several proangiogenic proteins, including the integrin subunit alpha5. Thus, miR-92a may serve as a valuable therapeutic target in the setting of ischemic disease.

MicroRNAs (miRs) are small noncoding RNAs that regulate a wide range of physiological and pathophysiological processes (*1*–*4*). The conserved miR-17~92 cluster, consisting of miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a (*3*), shows up-regulated expression in tumors (*5*–*7*), and its overexpression in tumor cells promotes tumor angiogenesis (*8*). The proangiogenic functions of the miRs encoded by the cluster have been attributed to miR-18a and miR-19a, which promote tumor angiogenesis by suppressing the release of soluble anti-angiogenic factors by tumor cells, thereby affecting endothelial cells (ECs) in a paracrine manner (*8*). Inhibition of miR processing by genetic knockdown of Dicer expression impairs EC functions and angiogenesis (*9*–*12*), and several miRs stimulate [let-7f (*11*), miR-27b (*11*), and miR-130 (*13*)] or inhibit [miR-221 and miR-222 (*14*)] angiogenesis in vitro or regulate vascular development [miR-126 (*15*, *16*)]. However, the postnatal function of individual endothelial miRs in vivo under pathophysiological conditions remains unclear (*17*).

Our microRNA expression profiling study revealed that human ECs express the miR-17~92 cluster, and particularly its member miR-92a (fig. S1), whose specific role in angiogenesis is unknown. In contrast to the proangiogenic effects of miR-18a and miR-19a in tumor angiogenesis (*8*), we found that forced overexpression of miR-92a in human ECs using a precursor molecule (termed pre92) [fig. S2A (*18*)] blocked sprout formation in a three-dimensional model of angiogenesis (Fig. 1, A and C), inhibited vascular network formation in matrigel assays (Fig. 1, B and C), reduced EC migration (fig. S2B), and impaired EC adhesion to fibronectin (Fig. 1D). Overexpression of miR-92a did not affect EC proliferation or viability (fig. S3). When human umbilical vein endothelial cells (HUVECs) were implanted in a matrigel plug in nude mice in vivo, pre92 reduced the number of invading cells and formation of in vivo perfused lectin-positive vessels (Fig. 1, E and F, and fig. S4A). In addition, the hemoglobin content (a surrogate marker of perfusion) was significantly lowered in the explanted matrigel plugs (fig. S4B). Likewise, overexpression of miR-92a induced severe defects in intersegmental vessel formation in zebrafish (fig. S5). These results indicate that overexpression of miR-92a blocks angiogenesis and vessel formation in vitro and in vivo.

We next investigated whether inhibition of miR-92a can enhance vessel growth. Indeed, inhibition of endothelial miR-92a in human ECs by 2′O-methyl antisense oligoribonucleotides increased sprout formation in vitro (fig. S6), leading us to test in vivo models. To inhibit miR-92a in vivo, we designed single-stranded RNA oligonucleotides complementary to specific miRs, also known as antagomirs, that are chemically modified for improved stability and cell delivery (*19*). Consistent with the hypothesis that inhibition of miR-92a augments angiogenesis, systemic application of antagomirs, specifically targeting miR-92a (antagomir-92a), enhanced the number of invading cells and in vivo perfused lectin-positive vessels in implanted matrigel plugs in mice (Fig. 2A and figs. S7 and S8). To control for off-target effects of single-stranded RNA oligonucleotides and to confirm the specificity of antagomir-92a, we used two different control antagomirs, neither of which affected plug vascularization (fig. S7).

To further characterize the in vivo role of miR-92a in angiogenesis, we used a mouse hindlimb ischemia model to study whether the expression level of miR-92a in skeletal muscle changes in response to ischemic injury. Ischemic injury significantly increased miR-92a expression, with a maximal effect seen within 1 to 3 days after injury (Fig. 2B). We next tested whether antagomir-92a can promote neovascularization in ischemic limbs. Mice systemically injected with antagomir-92a exhibited a significant reduction in toe necrosis in comparison with mice treated with a control antagomir or phosphate-buffered saline (PBS) (fig. S9). Recovery of blood flow was increased in the antagomir-92a–treated mice, consistent with the hypothesis that the reduced necrosis was due to improved neovascularization in the ischemic limb (Fig. 2C). Likewise, the number of capillaries and smooth muscle actin-positive arterioles was increased after antagomir-92a treatment (fig. S10), indicating that antagomir-92a improves perfusion and the functional recovery of ischemic limbs. To

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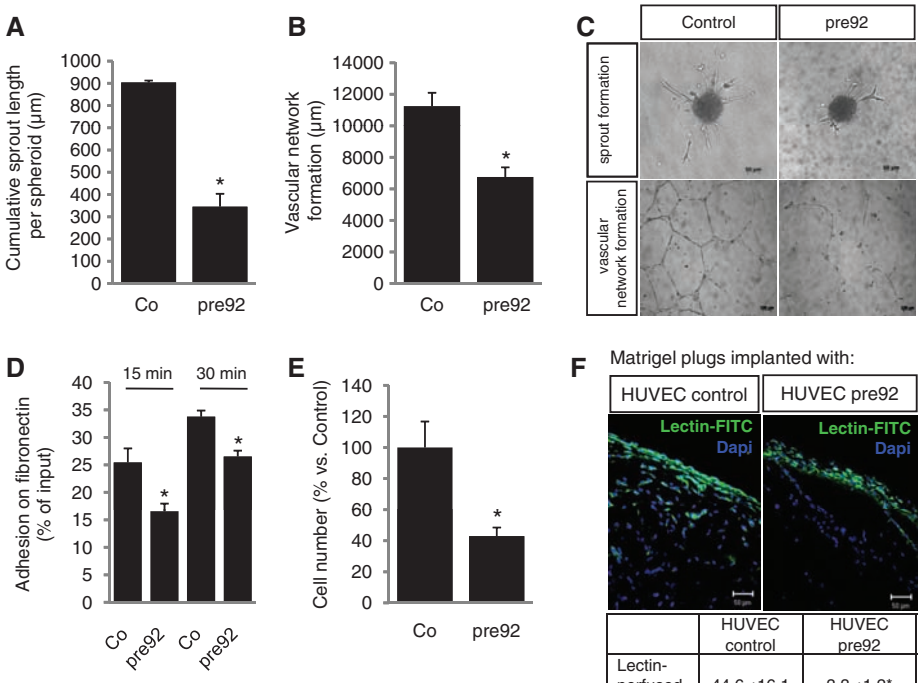
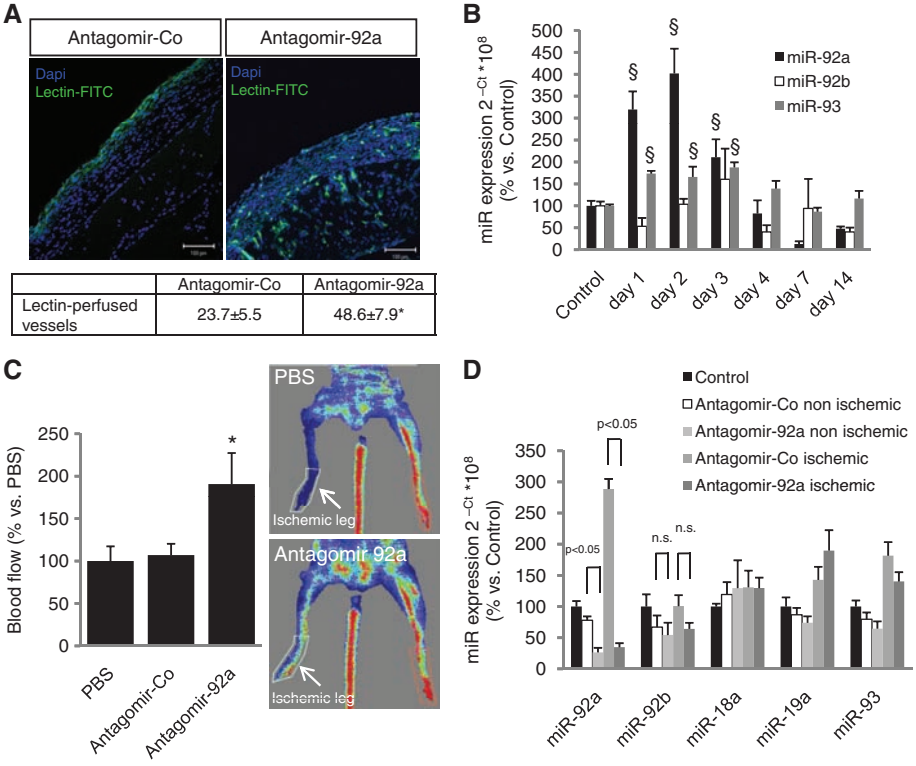
test the specificity of antagomir-92a, we studied its effect on the expression of miR-92a, the closely related miR-92b, and members of the miR-17~92 cluster, namely miR-18a and miR-19a, and the unrelated miR-93. Whereas a single intravenous injection of antagomir-92a in mice significantly suppressed miR-92a expression in nonischemic and ischemic limbs, this treatment only slightly and nonsignificantly reduced the expression of miR-92b and had no effect on the expression of the other miRs studied (Fig. 2D).

miR-92a also was up-regulated after induction of acute myocardial infarction (fig. S11). Therefore, we investigated the effect of antagomir-92a treatment on recovery of heart function in a mouse model of acute myocardial infarction. We intravenously injected antagomir-92a or a control antagomir at days 0, 2, 4, 7, and 9 after occlusion of the left coronary artery, and then determined left ventricular (LV) function by Millar catheterization at day 14. Compared with controls, antagomir-92a treatment improved LV systolic and diastolic function, as evidenced by increases of maximum and minimum rate of rise of left ventricular pressure (dp/dt_{max} and dp/dt_{min}), and significantly reduced end-diastolic pressure and tau (Fig. 3, A and B, fig. S12, and table S1). Furthermore, antagomir-



	HUVEC control	HUVEC pre92
Lectin-perfused vessels	44.6 ±16.1	3.3 ±1.2*

Fig. 1. Effect of overexpression of miR-92a on angiogenesis in human ECs. (**A**) Inhibition of sprout formation in spheroids (n = 10 spheroids per experiment, n = 3 experiments) and (**B**) matrigel assays in vitro (n = 3 experiments). Representative micrographs are shown in (**C**). (**D**) Effect of miR-92a overexpression on adhesion of ECs to fibronectin (n = 4 experiments). (**E** and **F**) HUVECs were transfected with pre92 or control pre-miR and 1 × 10^6 cells were implanted in matrigel plugs in vivo. (E) After 6 days, invading cells were detected in hematoxylin and eosin sections (n = 5 to 6 plugs). (F) Vessels were identified and counted after intravenous infusion of fluorescein isothiocyanate (FITC)–conjugated lectin (n = 8 high-power fields). Due to the implantation of HUVECs, baseline vascularization is higher compared with Fig. 2A. Data are mean ± SEM. *P < 0.05 versus control (Student's t test).



	Antagomir-Co	Antagomir-92a
Lectin-perfused vessels	23.7±5.5	48.6±7.9*

Fig. 2. Inhibition of miR-92a enhances angiogenesis and neovascularization in mice. (**A**) Effect of systemic infusion of antagomirs targeting miR-92a [8 mg per kg of body weight (mg/kg bw), n = 6 plugs] or control antagomirs (antagomir-Co, n = 5 plugs) at days 1, 3, and 5 in each mouse on the number of lectin-perfused vessels in matrigel plugs in vivo. *P < 0.05 versus antagomir-Co (t test). (**B**) Expression of miR-92a, miR-92b, and miR-93 was detected in ischemic muscle tissue compared with nonischemic control muscle at various days after ischemia. n = 3 mice each, §P < 0.05 versus control [analysis of variance (ANOVA)]. (**C**) miR-92a was inhibited by injecting antagomir-92a (8 mg/kg bw, injected at days 0, 2, 4, 7, and 9) after induction of hind-limb ischemia. At day 14, laser Doppler–derived blood flow was determined (n = 4 to 8 mice per group). *P < 0.05 versus PBS (t test). Representative images are shown in the right panel. (**D**) Antagomir-92a (8 mg/kg bw, injected intravenously once, directly after induction of ischemia) specifically reduced miR-92a expression in ischemic and nonischemic muscle tissue at 2 days after induction of hind-limb ischemia. Expression of miRs was determined by real-time polymerase chain reaction as indicated in antagomir-92a or control antagomir-treated mice and was compared with nontreated controls. n = 4 mice. Data are mean ± SEM (ANOVA).

92a treatment reduced the infarct size by 39 ± 15.5% ($P = 0.05$) (fig. S13), suppressed the number of apoptotic cells (fig. S14), and significantly augmented the number of in vivo perfused lectin-positive vessels, particularly in the infarct border zone (Fig. 3C and fig. S15). Antagomir-92a treatment also increased the number of smooth muscle actin-positive vessels (fig. S15). Control experiments for specificity confirmed that antagomir-92a suppressed the expression of miR-92a but did not affect expression of another miR (miR-24) in the heart (Fig. 3D). By using Cy3-labeled antagomir-92a to visualize biodistribution, we found that antagomir-92a was taken up by vascular cells, but also by other cells of the limb and heart (figs. S16 and S17). Consistently, antagomir-92a treatment inhibited miR-92a expression in the endothelium and in other cells of the heart, such as cardiac myocytes (figs. S18 to S20).

We next analyzed putative miR-92a targets predicted by defined criteria (*20*) using the TargetScan and miRanda software. The in silico predicted targets included mRNAs encoding several regulators of endothelial cell functions and vessel growth, such as the integrin subunits α5 and αv, which mediate cell-matrix interactions and cell migration (*21*–*23*). Additional predicted proangiogenic targets included the mRNAs encoding the histone deacetylase SIRT1 (*24*, *25*), the small guanosine triphosphate–binding protein Rap-1, an angiogenesis-mediating protein involved in integrin signaling (*26*), the sphingosine-1-phosphate receptor 1 ($S1P_1$) (*27*), and the mitogen-activated kinase kinase 4 (MKK4). Using an Affymetrix mRNA gene expression array, we confirmed that expression of the mRNAs for integrin subunits α5 (ITGA5) and αv, $S1P_1$, and MKK4 was reduced in response to miR-92a overexpression, whereas Rap-1 mRNA expression was unaffected in human ECs (Fig. 4A). Interestingly, the mRNA expression profile also revealed a down-regulation of endothelial NO-synthase (eNOS), which controls vascular tone and is essential for postnatal neovascularization (*28*, *29*). We confirmed that ITGA5 and eNOS protein levels were significantly reduced in HUVECs overexpressing miR-92a (Fig. 4B and fig. S21, A and B). Conversely, inhibiting miR-92a by antagomir-92a treatment in mice increased the expression of ITGA5 mRNA and protein in the vasculature of skeletal muscle tissue (figs. S21C and S22).

Given the pivotal role of ITGA5 in vascular development and angiogenesis (*21*, *22*) (fig. S23A) and its regulation by inhibition or overexpression of miR-92a, we investigated whether ITGA5 is a direct target of miR-92a. The 3′ untranslated region (3′UTR) of the ITGA5 mRNA contains one conserved predicted binding site for miR-92a (Fig. 4C). We therefore cloned a fragment of the ITGA5 3′UTR sequence downstream of a luciferase construct and examined luciferase activity after cotransfection with miR-92a in human embryonic kidney (HEK) cells. Indeed, miR-92a overexpression reduced luciferase activity (Fig. 4C), which strongly suggests that ITGA5 mRNA

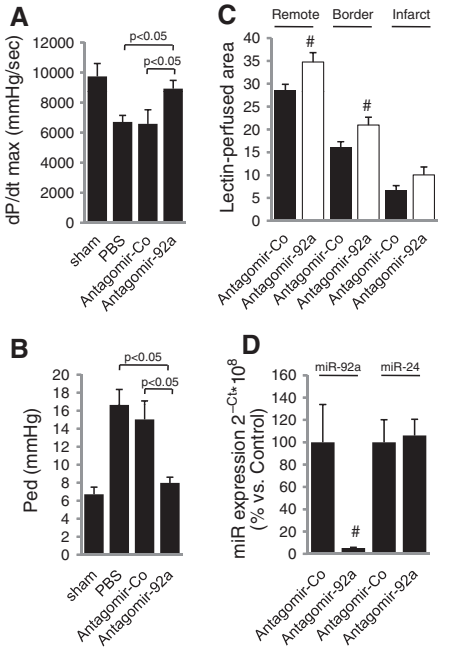


Fig. 3. Inhibition of miR-92a enhances recovery after acute myocardial infarction in mice. (**A** and **B**) 8 mg/kg bw antagomir-92a ($n = 8$ mice), control antagomirs ($n = 6$ mice), or PBS ($n = 7$ mice) were injected at days 0, 2, 4, 7, and 9 after induction of myocardial infarction. On day 14, cardiac catheterization was performed for functional analysis (dp/dt_{max} and end-diastolic pressure) compared with sham controls ($n = 5$ mice) (Mann-Whitney *U* test). (**C**) Capillary density was determined after intravenous infusion of FITC-conjugated lectin and was quantified in the remote, border, and infarct regions of the hearts by automatic quantification of lectin-positive pixels per total pixels ($\times 10^4$). Quantification of $n = 6$ high-power fields per region per group. #$P < 0.05$ versus antagomir-Co (*t* test). (**D**) miR-92a expression in hearts 6 days after antagomir-92a treatment ($n = 8$ mice) compared with control antagomirs ($n = 3$ mice). miR-24 expression was detected as control. Data are mean ± SEM. #$P < 0.05$ versus control antagomirs (Mann-Whitney *U* test).

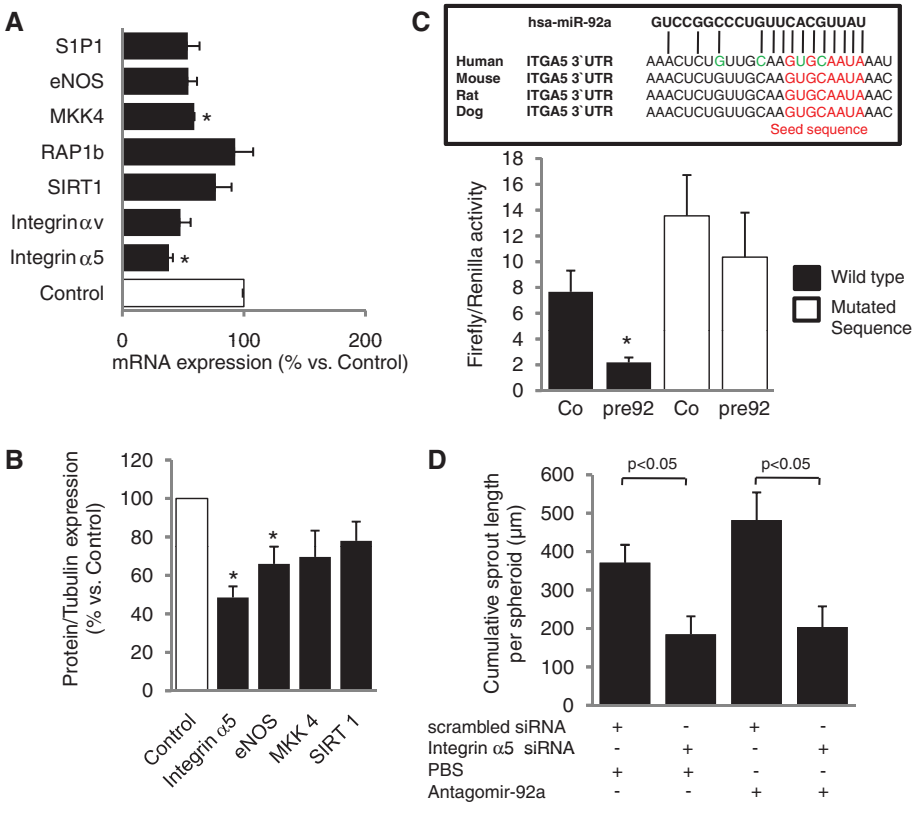


Fig. 4. Identification of miR-92a targets. (**A** and **B**) Expression analysis of genes regulated in pre-miR-92 overexpressing human ECs. (A) Results of Affymetrix mRNA expression profiles at 48 hours after pre92 transfection compared with controls ($n = 4$ to 5 experiments) (see also table S2). (B) Protein expression in human EC determined by Western blotting using antibodies against ITGA5, eNOS, MKK4, and SIRT1 ($n = 3$ to 4 experiments). ITGA5 down-regulation was confirmed by fluorescence-activated cell sorting (fig. S21). (**C**) (Top) Schematic illustration of miR-92a seed sequence in the integrin α5 3′UTR. Green indicates the mutated nucleotides. (Bottom) Luciferase normalized to Renilla activity measured in homogenates of HEK cells transfected with the wild-type or mutated luciferase constructs and pre-miR-92a or scrambled controls. Mutation of the target sequence resulted in a higher basal expression, indicating stabilization possibly by protecting against endogenous miR-92a. $n = 5$ to 6 experiments. (**D**) Effect of ITGA5 siRNA and antagomir-92a on spheroid sprout formation of HUVECs compared with scrambled siRNA, $n = 4$ experiments. The efficiency of ITGA5 siRNA is illustrated in fig. S25. Data are mean ± SEM. *$P < 0.01$ in (A), and $P < 0.05$ versus controls in all other panels (Student's *t* test).

is a direct target of miR-92a. Mutation of the target sequence prevented down-regulation of luciferase activity by pre-miR-92a (Fig. 4C). To determine whether the reduced expression of ITGA5 by miR-92a contributes to the inhibition of sprout formation and neovascularization, we inhibited ITGA5 expression by small interfering RNA (siRNA) in human ECs or used ITGA5$^{+/-}$ mice and found that antagomir-92a induced sprouting and that rescue of necrosis was reduced when ITGA5 was down-regulated, respectively (Fig. 4D and fig. S23B). Conversely, overexpression of ITGA5 partially rescued the miR-92a–mediated suppression of sprout formation in human ECs (fig. S24).

To explore whether the effect of miR-92a on mRNA expression levels can be mimicked by silencing ITGA5 expression, we compared the gene expression patterns in HUVECs overexpressing miR-92a versus HUVECs depleted of ITGA5. As predicted, several putative direct miR-92a targets such as MKK4 or $S1P_1$ were not affected by ITGA5 siRNA (table S2, yellow box). However, the expression levels of a second group of genes, which were down-regulated by miR-92a despite the lack of target sequences in their 3′UTR, were also reduced by siRNA against ITGA5 (table S2, red box). Hence, these genes might be secondarily regulated as a consequence of ITGA5 down-regulation. The most prominent example was the regulation of eNOS expression, which was down-regulated by miR-92a and ITGA5 siRNA, indicating that eNOS down-regulation in response to miR-92a overexpression may occur secondarily as a consequence of ITGA5 mRNA degradation.

We have identified miR-92a as an endogenous repressor of the angiogenic program in ECs, and we have shown in two mouse models that a reagent designed to inhibit the actions of this microRNA enhances the functional recovery of ischemic tissue. The mRNA encoding integrin subunit alpha5 is one of several candidate targets of miR-92a. The relevance of this potential target is evidenced by the severe vascular defects seen in mice genetically deficient in this integrin subunit (*21*). Although our studies have focused on ECs, we cannot formally exclude the possibility that miR-92a has effects on other cell types in the cardiovascular system. Indeed, apoptosis in the heart was reduced by antagomir-92a treatment. However, antagomir-92a did not affect apoptosis of cardiomyocytes in vitro (fig. S14B), suggesting that the antiapoptotic activity of antagomir-92a in vivo is mediated by an indirect mechanism. The capacity of miR-92a to target various downstream effectors might offer a therapeutic advantage to interfere with the complex modulation of vessel growth, maturation, and functional maintenance in ischemic diseases.

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Supporting Online Material

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Mitochondrial STAT3 Supports Ras-Dependent Oncogenic Transformation

Daniel J. Gough,[1]* Alicia Corlett,[1]*† Karni Schlessinger,[1]‡ Joanna Wegrzyn,[2] Andrew C. Larner,[2] David E. Levy[1]§

Signal transducer and activator of transcription 3 (STAT3) is a latent cytoplasmic transcription factor responsive to cytokine signaling and tyrosine kinase oncoproteins by nuclear translocation when it is tyrosine-phosphorylated. We report that malignant transformation by activated Ras is impaired without STAT3, in spite of the inability of Ras to drive STAT3 tyrosine phosphorylation or nuclear translocation. Moreover, STAT3 mutants that cannot be tyrosine-phosphorylated, that are retained in the cytoplasm, or that cannot bind DNA nonetheless supported Ras-mediated transformation. Unexpectedly, STAT3 was detected within mitochondria, and exclusive targeting of STAT3 to mitochondria without nuclear accumulation facilitated Ras transformation. Mitochondrial STAT3 sustained altered glycolytic and oxidative phosphorylation activities characteristic of cancer cells. Thus, in addition to its nuclear transcriptional role, STAT3 regulates a metabolic function in mitochondria, supporting Ras-dependent malignant transformation.

Signal transducers and activators of transcription (STATs) mediate cellular differentiation, proliferation, survival, and immune function (*1*). Tyrosine-phosphorylated STATs undergo phosphotyrosine–Src homology 2 (SH2) domain interactions, leading to nuclear translocation and expression of target genes (*2*). STAT3 mediates acute phase responses to cytokines such as interleukin-6 (*3*–*6*) and has been linked to cancer development (*7*). Deregulated tyrosine kinase oncoproteins can target constitutive STAT3 phosphorylation (*8*), resulting in gene expression associated with tumor cell survival and proliferation. Augmented STAT3 activity is associated with multiple human tumors, and STAT3 inhibition can mediate tumor regression (*9*). However, additional STAT3 functions that are inconsistent with functioning solely as a transcription factor have been described (*10*).

Ablation of STAT3 impairs malignant transformation by protein tyrosine kinase oncoproteins, such as anaplastic lymphoma kinase (ALK) and v-Src (*11*, *12*). To determine whether oncogenes that lack tyrosine kinase activity also require STAT3, we examined cellular transformation by activated Ras (H-RasV12, where V12 indicates valine-12) (*13*). Although previous evidence suggested that Ras

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